Quantification of Carotenoids in Citrus Fruit by LC-MS and Comparison of Patterns of Seasonal Changes for Carotenoids among Citrus Varieties

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To quantify the 18 carotenoids on the basic routes of the carotenoid biosynthesis in plants simultaneously, a method for liquid chromatography−mass spectrometry (LC-MS) using atmospheric pressure chemical ionization was developed. With this method, the seasonal changes of carotenoids in the flavedo and juice sacs of 39 citrus varieties were analyzed. On the basis of the patterns of seasonal changes of carotenoids in both flavedo and juice sacs, 39 citrus varieties were classified. In flavedo, 39 varieties were classified into 5 clusters, in which the carotenoid profiles were carotenoid-poor, phytoene-abundant, violaxanthin-abundant, violaxanthin- and β-cryptoxanthin-abundant, and phytoene-, violaxanthin-, and β-cryptoxanthin-abundant, respectively. In juice sacs, they were classified into 4 clusters, in which the carotenoid profiles were carotenoid-poor, violaxanthin-abundant, violaxanthin- and phytoene-abundant, and violaxanthin-, phytoene-, and β-cryptoxanthin-abundant, respectively. In flavedo, many citrus varieties, except for the carotenoid-poor and phytoene-abundant varieties, massively accumulated β,ϵ-carotenoids (e.g., lutein), β,β-carotenoids (e.g., β-cryptoxanthin and violaxanthin), and phytoene, in that order. In juice sacs, the accumulation order among β,β-carotenoids was observed. Violaxanthin accumulation preceded β-cryptoxanthin accumulation in violaxanthin-, phytoene-, and β-cryptoxanthin-abundant varieties. In each variety, the carotenoid profiles of the flavedo and juice sacs on the basis of the concentration in violaxanthin and β-cryptoxanthin were similar, with the exception of a few varieties.

KEYWORDS: Citrus; carotenoids; LC-MS; seasonal change; classification

INTRODUCTION

Carotenoids are important components for fruit quality. In citrus fruit, peel and juice colors are mainly due to the presence of these pigment (*1*). In addition, some carotenoids serve as precursors for vitamin A, which is essential to human and animal diets, and as antioxidants, which play a role in reducing the risk of chronic diseases (*2, 3*). The pathway of carotenoid biosynthesis in plants has been extensively studied (*4−9*). The first committed step of carotenoid biosynthesis is the condensation of two molecules of geranylgeranyl pyrophosphate (C_{20}) to form a colorless phytoene (C_{40}). Phytoene desaturase and ζ-carotene desaturase convert phytoene into lycopene via phytofluene, ζ-carotene, and neurosporene. The cyclization of lycopene to yield α-carotene with the ϵ- and β-rings via δ-carotene and β-carotene with two β-rings via γ-carotene is a crucial branching point in the pathway. α-Carotene is converted into lutein by sequential hydroxylation of the ϵ- and β-rings. Furthermore, lutein is converted into lutein epoxide by epoxidation of the β-ring. β-Carotene is converted to zeaxanthin via

β-cryptoxanthin by a two-step hydroxylation. Zeaxanthin is converted into violaxanthin via antheraxanthin by sequential epoxidation of two β-rings. Furthermore, violaxanthin is converted into neoxanthin by rearrangement of one epoxy ring of violaxanthin to form an allenic bond.

Citrus is a complex source of carotenoids, with the largest number of carotenoids found in any fruit (*1*). Generally, high-performance liquid chromatography (HPLC) with a photodiode array detector and a C_{30} column has been used for the study of citrus carotenoids (*10−13*). The method is convenient and reliable for carotenoid analysis. However, several problems sometimes occur in citrus carotenoid analysis with this method because of the complicated composition of carotenoids in citrus fruit. Previously, several citrus carotenoids (phytoene, ζ-carotene, α-carotene, lutein, β-carotene, β-cryptoxanthin, zeaxanthin, and violaxanthin) on the basic routes of the carotenoid biosynthesis in plants were analyzed by HPLC to investigate the relationship between the accumulation of carotenoids and the expression of carotenoid biosynthetic genes (*13*). However, separation of zeaxanthin from other carotenoids in citrus fruit and separation of β-carotene from ζ-carotene isomers were not

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performed with a single HPLC analysis. To eliminate these problems in the HPLC method with the C_{30} column, three different gradient elution schedules were used (*13*). These gradient elution schedules were effective for the specific detection of zeaxanthin, β-carotene, and ζ-carotene in citrus. However, the method required a long time because a sample must be analyzed with three different gradient elution schedules. In addition, some carotenoids, such as phytofluene and antheraxanthin, on the basic routes of the carotenoid biosynthesis were not quantified with this method because of the insufficient sensitivity of the detector and the interference from impurities in extracts. Thus, to investigate the seasonal changes of carotenoids in citrus fruit, a new analytical method for carotenoids that would have sufficient sensitivity, selectivity, and capacity to analyze a large number of samples was required. There have been several reports on liquid chromatography—mass spectrometry (LC-MS) methods for carotenoid analysis in a complex matrix, such as serum or eggs (*14−18*). Moreover, in citrus fruit, the high selectivity and sensitivity of mass spectrometric detection would make it possible to quantify carotenoids clearly and rapidly. Therefore, in the present study, an LC-MS method for the simultaneous quantification of 18 carotenoids on the basic routes of the carotenoid biosynthesis in plants was developed. The method made it possible to analyze several citrus carotenoids that were difficult to quantify by a single HPLC analysis with UV−vis absorbance detection and was routinely applied to the present analyses.

Several reports have indicated that differences in the carotenoid composition can be used to classify citrus fruit. Goodner et al. (*19*) reported that the difference in the concentration of β-cryptoxanthin in juice can be used as a discriminating factor among mandarin, orange, and their hybrids. Fanciullino et al. (*12*) reported that *cis*-violaxanthin and β-cryptoxanthin in juice were strong determinants in the classification on the 25 citrus genotypes. Thus, these studies focused on the diversity of the carotenoid profiles at a stage of fruit ripening. On the other hand, in these studies, the difference in the seasonal changes of carotenoids among citrus varieties was not investigated. Neither was the diversity of the carotenoid in flavedo. Kato et al. (*13*) compared the seasonal changes in the carotenoid contents and gene expression of carotenoid-biosynthetic enzymes among three citrus varieties, Satsuma mandarin, Valencia orange, and Lisbon lemon, in both flavedo and juice sacs. The study showed that the carotenoid accumulation during fruit ripening was highly regulated by the coordination of the expression among carotenoid biosynthetic genes and that the seasonal changes of carotenoids during ripening were diverse among Satsuma mandarin, Valencia orange, and Lisbon lemon. The study also showed that a pathway change from the accumulation of β,ϵ-carotenoids, that is, carotenoids with one β-ring and an ϵ-ring, to that of β,β-carotenoids, that is, carotenoids with two β-rings, occurred with the transition of peel color from green to orange in the flavedo of Satsuma mandarin and Valencia orange. These findings implied that the difference in the seasonal changes of carotenoids during ripening can also be used to classify citrus fruit. However, their study compared the patterns of seasonal changes of carotenoids among only three citrus varieties.

In the present study, an LC-MS method for the simultaneous quantification of 18 carotenoids on the basic route of the carotenoid biosynthesis in plants was developed. With this method, seasonal changes of carotenoids in flavedo and juice sacs of 39 citrus varieties during ripening were analyzed. On the basis of the patterns of seasonal changes of carotenoids in

Table 1. Citrus Varieties Used in the Present Study

scientific name	conventional name	sample no.
section Limonellus		
C. latifolia Tanaka	Tahiti lime	A-1
C. limettioides Tanaka	sweet lime	A-2
section Citrophorum		
C. limon Burm. f.	Eureka lemon	B-1
C. limonia Osbeck	Limonia	B-2
C. limonia Osbeck	Rangpur lime	B-3
section Cephalocitrus		
C. grandis Osbeck	Hirado buntan	C-1
C. paradisi Macf.	red blush grapefruit	C-2
C. paradisi Macf.	Marsh grapefruit	C-3
C. glaberrima Hort. ex Tanaka	Kinukawa	C-4
C. intermedia Hort. ex Tanaka	Yamamikan	C-5
C. hassaku Hort. ex Tanaka	Hassaku	C-6
C. tengu Hort. ex Tanaka	Tengu	C-7
section Aurantium		
C. medioglobosa Hort. ex Tanaka	Naruto	D-1
C. natsudaidai Hayata	Natsudaidai	D-2
C. rugulosa Hort. ex Tanaka	Attani	D-3
C. rokugatsu Hort. ex Y. Tanaka	Rokugatsumikan	D-4
C. canaliculata Hort. ex Y. Tanaka	Kikudaidai	D-5
C. sinensis Osbeck	Trovita orange	D-6
C. sinensis Osbeck	Washington navel orange	D-7
C. tankan Tanaka	Tankan	D-8
C. iyo Hort. ex Tanaka	Iyo	D-9
C. tamurana Hort. ex Tanaka	Hyuganatsu	D-10
C. ujukitsu Hort. ex Tanaka	Ujukitsu	D-11
C. aurea Hort. ex Tanaka	Kawabata	D-12
C. shunkokan Hort. ex Tanaka	Shunkokan	D-13
section Osmocitrus		
C. junos Sieb. ex Tanaka	Yuzu	E-1
C. pseudo-aurantium Hort. ex Y. Tanaka	Henkamikan	E-2
section Acrumen		
C. nobilis Lour.	Kunenbo	F-1
C. unshiu Marc.	Satsuma mandarin	F-2
C. yatsushiro Hort. ex Tanaka	Yatsushiro	F-3
C. keraji Hort. ex Tanaka	Kabuchi	F-4
C. reticulata Blanco	Ponkan	F-5
C. deliciosa Ten.	Mediterranean mandarin	F-6
C. tangerina Hort. ex Tanaka	Obenimikan	F-7
C. tangerina Hort. ex Tanaka	Dancy tangerine	F-8
C. tachibana Tanaka	Tachibana	F-9
C. kinokuni Hort. ex Tanaka	Hirakishu	F-10
C. reshni Hort. ex Tanaka	Cleopatra	F-11
C. depressa Hayata	Shiikuwasha	F-12

both flavedo and juice sacs, 39 citrus varieties were classified. The present study was the first of its kind, to our knowledge, to investigate the characteristics in the patterns of the seasonal changes of carotenoids using many citrus varieties belonging to sections Limonellus, Citrophorum, Cephalocitrus, Aurantium, Osmocitrus, and Acrumen in the genus *Citrus*. In addition, the present study is the first of its kind, to our knowledge, to compare the carotenoid profiles between flavedo and juice sacs using many citrus varieties.

MATERIALS AND METHODS

Plant Material. The classification and nomenclature of citrus varieties were based on Tanaka Systematics (*20*). Fruit of citrus varieties listed in **Table 1** were harvested from trees at the National Institute of Fruit Tree Science, Okitsu Citrus Research Station, Okitsu (Shizuoka, Japan). These were collected monthly from late October to late February. On each sampling date, at least three homogeneous fruits for each variety were collected from the same tree. The flavedo and juice sacs were separated from sampled fruit. In the separation of flavedo, albedo, the spongy material coating the inner surface of the flavedo, was chipped off using a kitchen knife so as not to break the oil gland in the flavedo. In juice sacs, the segment walls, that is, the outer surface of the segments, were chipped off using a razor. The

samples were immediately frozen in liquid nitrogen and kept at −80 °C until use. Under these conditions, the carotenoids observed in this study were stable in the samples for at least 2 years.

Chemicals. *all-trans*-α-Carotene (β,ε-carotene), *all-trans*-β-carotene (β,β-carotene), and *all-trans*-lycopene (ψ,ψ-carotene) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *all-trans*-Antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol) and *all-trans*-neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol) were obtained from DHI Water and Environment (Horsholm, Denmark). *all-trans*-Zeaxanthin (β,β-carotene-3,3′-diol) was obtained from Extrasynthese (Genay, France). *all-trans*-β-Cryptoxanthin (β,β-caroten-3-ol) was obtained from Sokenkagaku (Tokyo, Japan). *all-trans*-Neurosporene (7,8-dihydro-ψ,ψ-carotene), *all-trans*-γ-carotene (β,ψ-carotene), *all-trans*-lutein epoxide (5,6-epoxy-5,6-dihydro-β,ε-carotene-3,3′-diol), *all-trans*-violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol), and *all-trans*-mutatoxanthin (5,8-epoxy-5,8-dihydro-β,β-carotene-3,3′-diol) were obtained from Carote-Nature (Lupsingen, Switzerland). *all-trans*-Lutein (β,ε-carotene-3,3′-diol) was purchased from Fluka Chemie (Buchs, Switzerland). Phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene), phytofluene (7,8,11,-12,7′,8′-hexahydro-ψ,ψ-carotene), three isomers of ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene), *cis*-violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol), and *cis*-antheraxanthin were isolated from citrus flavedo and tentatively identified according to the previously reported methods (*13, 21−26*). These carotenoids were used as authentic standards. β-Apo-8′-carotenal was purchased from Fluka Chemie. Potassium hydroxide (KOH), 2,6-di-*tert*-butyl-4-methylphenol (BHT), diethyl ether, HPLC grade methyl *tert*-butyl ether (MTBE), methanol (MeOH), and other chemicals were purchased from Wako Pure Chemical Ind., Ltd.

Preparation of the Internal Standard, Citranaxanthin. Citranaxanthin was synthesized and identified according to the procedures described by Stewart et al. (*27*). Briefly, 0.5 mL of acetone and 1 mL of 10% methanolic KOH were added into a solution of β-apo-8′-carotenal (0.3 mmol in 10 mL of diethyl ether/methanol, 1:1) at 0 °C. The mixture was stirred for 18 h in the dark at room temperature in an argon atmosphere. The mixture was transferred to a separatory funnel containing 100 mL of diethyl ether. The ether phase was washed five times with NaCl-saturated water to remove KOH and treated with anhydrous Na_2SO_4. After the ether phase was evaporated, the residue was purified using silica gel column chromatography using chloroform/MeOH (40:0.5, v/v). The dark red crystal of the product was obtained (122 mg). The product was analyzed by high-resolution fast atom bombardment mass spectrometry (HRMS-FAB) (Mstation-700 double-focusing magnetic sector mass spectrometer, JMS-700, JEOL, Tokyo, Japan) and UV−vis spectrophotometry (UV-2200 spectrophotometer, Shimadzu, Kyoto, Japan) and identified as citranaxanthin: UV−vis (hexane) λ_{max}, nm, 493, 464; HRMS-FAB (*m/z*) [M + H]$^+$ calcd for $C_{33}H_{45}O$, 457.3470; found, 457.3452.

Carotenoid Extraction. Samples (ca. 3 g of flavedo and ca. 5 g of juice sacs) were homogenized in 50 mL of 40% (v/v) aqueous methanol containing 0.5 g of basic magnesium carbonate using a homogenizer (Polytron homogenizer T-25). After vacuum filtration, the pigment in the residue was extracted with diethyl ether/MeOH (7:3, v/v) containing 0.1% (w/v) BHT until the residue was colorless. The extract was transferred to a separatory funnel containing 100 mL of diethyl ether. The ether phase was washed two times with NaCl-saturated water and treated with anhydrous Na_2SO_4. After the ether phase was evaporated, the residue was dissolved with 10 mL of diethyl ether, mixed with 5 mL of 20% methanolic KOH, and placed for 18 h in the dark in an argon atmosphere at room temperature. The alkaline mixture was transferred to a separatory funnel containing 100 mL of diethyl ether. The ether phase was washed five times with NaCl-saturated water and treated with anhydrous Na_2SO_4. After the ether phase was evaporated, the residue was transferred to a 5 mL volumetric flask in which the volume was adjusted with MTBE/MeOH (1:1, v/v) containing 0.1% BHT to 5 mL. The sample was filtered through a 0.2 μm filter (Millipore, Bedford, MA) and stored at −30 °C before injection into the LC-MS. Five microliters of the solution was injected.

Chromatographic Conditions. The separation of carotenoids was carried out using a C_{30} carotenoid column (250 × 4.6 mm i.d., 5 μm,

YMC, Kyoto, Japan) and ternary gradient elution with water (eluent A), MeOH (eluent B), and MTBE (eluent C) as mobile phases at a flow rate of 1 mL/min. The column temperature was kept at 25 °C. The linear gradient program was performed as follows: the initial condition was 5% A/90% B/5% C; 0−12 min, 95% B/5% C; 12−20 min, 86% B/14% C; 20−30 min, 75% B/25% C; 30−50 min, 50% B/50% C; 50−60 min, 37.5% B/62.5% C; 60−73 min, 5% B/95% C.

LC-MS Conditions. Analyses were performed on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) consisting of a quaternary pump, an online degasser, a photodiode array detector (set to scan 220−550 nm), and a temperature-controlled autosampler (set at 4 °C) coupled to a PE SCIEX API2000 triple-stage quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA) with a heated nebulizer ionization source. The analytes were detected using atmospheric pressure chemical ionization (APCI) in a positive mode. The curtain gas (nitrogen), nebulizer gas (air), and auxiliary gas (air) were used at pressures of 45, 60, and 20 psi, respectively. The ion source temperature was maintained at 400 °C. The nebulizer current was set at 1 μA. The quantification of carotenoids was performed in the selected ion monitoring (SIM) mode with a dwell time of 77 ms. The Q-pole parameter (DP, EP, FP) was optimized for each carotenoid. Data collection and peak integration were performed using Analyst software (version 1.4, Applied Biosystems).

Quantification and Method Validation. To correct the response variation in the mass spectrometer, the internal standard, citranaxanthin, was added into all standard solutions and citrus extracts just before the solutions were injected to LC-MS. The final concentration of the internal standard was 0.14 μg/mL. Calibration curves were constructed using standard solutions of the carotenoids at five different concentrations in MTBE/MeOH (1:1, v/v). The linearity of the calibration curve was confirmed by plotting the peak area ratio of the analyte to the internal standard versus the concentration. The intraday and interday precisions were calculated by replicate analyses (*n* = 6) of the same sample and expressed as the coefficient of variation (CV %). The accuracy of the method was calculated by (observed concentration/known concentration) × 100. The recovery of each carotenoid during the extraction was determined according to the ratio of the amount added to that measured experimentally after the extraction. The assessment of the matrix effect derived from the difference in the varieties in carotenoid analysis was carried out according to the method of Matuszewski et al. (*28*). Matrix-matched calibration curves were constructed using the flavedo and juice sacs of five varieties. By comparing the slopes of the calibration curves, the absence and presence of a matrix effect were assessed. The lower limit of quantification (LLOQ) for each analyte was determined with a signal-to-noise ratio of 10:1.

Cluster Analysis. Hierarchical clustering was performed by Ward's method in the application software JMP 6 (JMP release 6.0; SAS Institute Inc., Cary, NC). First, to group the pattern of seasonal changes in each carotenoid on the basis of their similarity, the carotenoid concentrations from October to January of 39 varieties were standardized and, subsequently, used for cluster analysis. In most carotenoids, the cluster analysis classified the patterns of seasonal changes into three groups, H, M, and L, in which carotenoid concentrations were high, medium, and low, respectively. Second, to classify citrus varieties on the basis of the patterns of seasonal changes of carotenoids, the categories of the patterns of the seasonal changes, H, M, and L, were converted into numerical values (ordinal scale), that is, 3, 2, and 1, respectively. By cluster analysis, 39 varieties were classified on the basis of similarity in the numerical values for the patterns of seasonal changes in phytoene, β-cryptoxanthin, and violaxanthin. A previous study (*12*) showed that two carotenoids, *cis*-violaxanthin and β-cryptoxanthin, were strong determinants in citrus classification. In the present study, the concentrations of not only the two carotenoids but also phytoene were higher than those of others, and the difference in the concentrations among the varieties was larger than that in other carotenoids. Thus, three carotenoids, phytoene, β-cryptoxanthin, and violaxanthin, were selected to classify the citrus varieties.

RESULTS

Identification of Carotenoids in Citrus. The identification of the carotenoids in flavedo and juice sacs was carried out using

Table 2. Chromatographic and Spectroscopic Characteristics of Citrus Carotenoids in Juice Sacs of Tankan in December

peak no.	m/z	retention time (min)	λ_{max} (nm, online) in citrus extract	λ_{max} (nm, online) standard	epoxide test: hypsochromic shift (in EtOH) before test	after test	identification
1	545.8	26.3	274, 286, 300	274, 286, 300			phytoene[c]
2	543.4	31.4	332, 348, 368	332, 348, 366			phytofluene[c]
3	541.7	39.2	380, 400, 424	380, 400, 424			ζ-carotene isomer 1[c]
4	541.7	40.9	380, 400, 424	380, 400, 424			ζ-carotene isomer 2[c]
5	541.7	42.3	380, 400, 424	380, 400, 424			ζ-carotene isomer 3[c]
6	537.4	35.8	418, 446, 472	418, 446, 472			*all-trans*-α-carotene
7	537.4	39.4	422, 450, 478	424, 450, 476			*all-trans*-β-carotene
8	553.3	31.5	422, 450, 478	422, 450, 478			*all-trans*-β-cryptoxanthin
9	569.4	23.6	423, 450, 474	423, 450, 476			*all-trans*-zeaxanthin
10	551.7	20.9	420, 442, 472	420, 442, 472			*all-trans*-lutein
11	585.8	15.1 (23.7)[a]	416, 436, 468[a]	416, 438, 468[a]	415, 440, 469	398, 421, 448	*all-trans*-lutein epoxide
12	585.8	17.7 (32.4)[a]	444, 472[a]	444, 472[a]	418, 445, 472	402, 427, 452	*all-trans*-antheraxanthin
13	585.8	20.2	402, 428, 450	400, 426, 450			mutatoxanthin isomer 1
14	585.8	21.3	402, 428, 450	400, 426, 450			mutatoxanthin isomer 2
15	585.8	24.5 (31.5)[b]	332, 440, 468[b]	332, 440, 468[b]	416, 441, 468	401, 424, 450	*cis*-antheraxanthin[c]
16	601.4	12.4	414, 438, 468	414, 438, 468	417, 440, 470	378, 400, 426	*all-trans*-violaxanthin
17	601.4	13.2 (19.4)[a]	412, 434, 462[a]	412, 434, 462[a]	413, 436, 466	399, 422, 449	*all-trans*-neoxanthin
18	601.4	17.5 (30.7)[a]	326, 410, 434, 462[a]	326, 410, 434, 462[a]	413, 437, 465	380, 400, 427	*cis*-violaxanthin[c]

[a] The chromatographic conditions were as follows: ternary gradient elution with water (eluent A), MeOH (eluent B), and MTBE (eluent C) as the mobile phase at a flow rate of 1 mL/min. The column temperature was kept at 25 °C. The initial condition was 4% A/95% B/1% C; 30–90 min, 4% A/6% B/90% C. The retention times in parentheses were observed in the method. [b] A chromatographic condition reported by Meléndez-Martínez et al. (*24*) was used. The retention time in parentheses was observed in the method. [c] Standard compounds isolated and tentatively identified were used for the identification. For the identification of all other compounds, authentic standards were used.

LC-DAD-MS on the basis of the comparison of the retention time, mass and UV–vis absorption spectra, and cochromatography with authentic or tentatively identified standards isolated from citrus (*25*, *26*). The flavedo of Mediterranean mandarin in December and juice sacs of Tankan in December, which have the most complex carotenoid profiles, were used for carotenoid identification. A chemical test (acid-catalyzed epoxide–furanoid oxide rearrangement: epoxide test) was also conducted to support the identification (*29*). **Table 2** shows the chromatographic and spectroscopic characteristics of carotenoids extracted from flavedo and juice sacs. The carotenoids, with the exception of lutein, formed an $[M + H]^+$ ion as a base peak on the mass spectra. Lutein formed an $[M - H_2O + H]^+$ ion at *m/z* 551 as a base peak and did not form an $[M + H]^+$ ion (**Table 2**). Previous studies indicated that the allyl alcohol moiety of lutein was easily dehydrated by β-elimination and converted to dehydrolutein in the APCI vaporizer and, subsequently, converted to the $[M - H_2O + H]^+$ ion (*16*, *30*). Therefore, the $[M - H_2O + H]^+$ ion for lutein and the $[M + H]^+$ ion for other carotenoids were used in the present study.

Figure 1 shows the typical LC-DAD-MS chromatograms of carotenoids extracted from juice sacs of Tankan in December. A total of 18 carotenoids were resolved by LC-MS, in which the isobaric compound and geometrical isomers in the carotenoid extract were strictly separated because these compounds have the same molecular weight. The online UV–vis spectra of the peaks, with the exception of peaks 11, 12, 17, and 18, agreed well with the standards in the present method. The spectra of peaks 11, 12, 17, and 18 were inconsistent with the spectra of the standards because those peaks overlapped with other carotenoids or impurities. Therefore, peaks 11, 12, 17, and 18 were fractionated and analyzed by other gradient methods (**Table 2**). Under the conditions, peaks 11, 12, 17, and 18 were strictly separated, and the UV–vis spectra of the peaks were consistent with each carotenoid standard (**Table 2**). Thus, peaks 1–14 and 16–18 were identified in **Table 2** on the basis of their UV–vis spectra. The identification for these compounds was supported by cochromatography with authentic standards

or tentatively identified standards on mass and UV–vis chromatograms.

Peak 15 showed the spectrometric characteristics of *cis*-antheraxanthin reported by Meléndez-Martínez et al. (*24*). Because the authentic standard for *cis*-antheraxanthin was not available, the peak was fractionated and analyzed using the method of Meléndez-Martínez et al. (*24*). The retention time and mass and absorption spectra of the peak were consistent with the reported values (*24*). Therefore, peak 15 was tentatively identified as *cis*-antheraxanthin.

Furthermore, the fractionated peaks 11, 12, and 15–18 were applied to an epoxide test to confirm the presence of one or two 5,6-epoxide groups in the structure (*29*). The hypsochromic shifts of about 20 nm for peaks 11, 12, 15, and 17 and about 40 nm for peaks 16 and 18 observed after the addition of 0.1 N ethanolic HCl showed the presence of one or two 5,6-epoxide groups in the structures (**Table 2**). The absorption maxima of carotenoid standards after the addition of HCl were also consistent with those of the fractionated peaks. These results also supported the identification of the peaks.

In flavedo of Mediterranean mandarin in December, all peaks, with the exception of peaks 13 and 14 (mutatoxanthin isomers), were detected and identified in the same manner as those in juice sacs. During routine analysis, the peaks were identified by the retention times on mass chromatograms.

Construction of LC-MS Method for Carotenoid Quantification. Quantification methods for the 18 carotenoids shown in **Table 3** were constructed by LC-MS using selected ion monitoring (SIM). In the present study, quantification methods for *cis*-antheraxanthin and mutatoxanthin isomers were not constructed because the amount of standards was insufficient to construct them. Quantification was performed according to an internal standard method. Citranaxanthin was selected as an internal standard because it has been reported to be absent in citrus fruit (*27*). Citranaxanthin formed the $[M + H]^+$ ion at *m/z* 457 as a base peak under the present condition. The peak of citranaxanthin eluted at 30.2 min and did not overlap with target carotenoids and isobaric impurities extracted from citrus on the mass chromatogram of *m/z* 457. **Table 3** shows that the
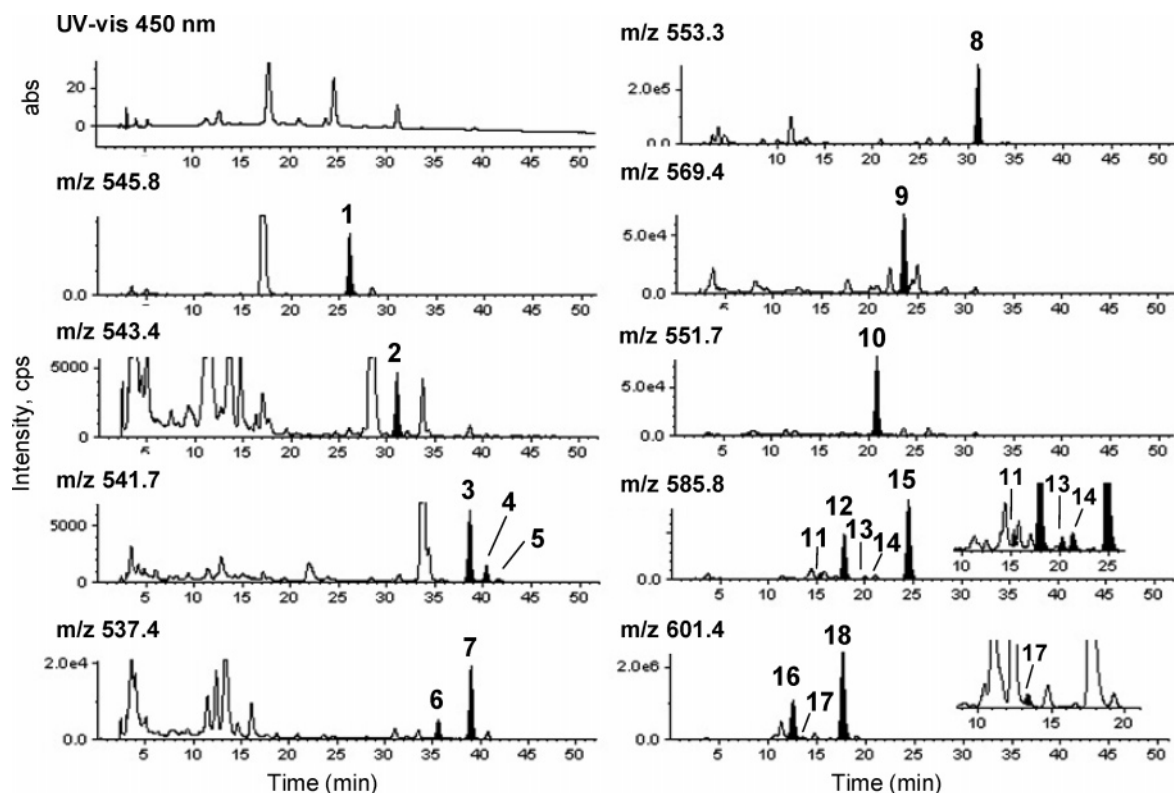
Figure 1. Typical LC-DAD-MS chromatograms of carotenoid in citrus juice sacs of Tankan (D-8) in December.

Table 3. Carotenoids Quantified by LC-MS and the Validation Data

carotenoid	m/z	retention time (min)	calibration linearity (r^2)	precision (CV %) intraday	precision (CV %) interday	recovery (%)	accuracy (%)	LLOQ[a] LC-MS	LLOQ[a] HPLC UV−vis
acyclic carotenoids									
phytoene	545.8	26.3	0.997	1.5	3.3	107	95	0.23	6.8
phytofluene	543.4	31.4	0.999	1.5	2.6	85	99	0.08	9.1
ζ-carotene isomer 1	541.7	39.2	0.999	1.5	2.3	93	98	0.07	5.5
ζ-carotene isomer 2	541.7	40.9	0.991	1.0	2.8	93	90	0.05	5.5
ζ-carotene isomer 3	541.7	42.3	0.998	2.5	2.3	95	98	0.22	5.5
all-trans-neurosporene	539.8	53.9	0.981	3.1	3.2	102	95	1.00	100.0
all-trans-lycopene	537.4	65.5	0.993	4.8	3.0	92	90	2.30	23.2
β,ε-carotenoids									
all-trans-α-carotene	537.4	35.8	0.994	2.6	4.9	100	94	0.04	1.0
all-trans-lutein	551.7	20.9	0.999	1.2	2.6	84	94	0.44	14.0
all-trans-lutein epoxide	585.8	15.1	0.999	1.5	5.4	97	106	0.07	12.0
β,β-carotenoids									
all-trans-γ-carotene	537.4	53.0	0.997	2.8	2.0	90	98	1.20	22.5
all-trans-β-carotene	537.4	39.4	0.999	3.8	4.4	107	97	0.08	32.7
all-trans-β-cryptoxanthin	553.3	31.5	0.999	1.3	3.4	95	95	0.12	16.8
all-trans-zeaxanthin	569.4	23.6	0.991	3.5	2.6	98	95	0.11	6.8
all-trans-antheraxanthin	585.8	17.7	0.995	1.3	3.0	97	103	0.16	12.9
all-trans-violaxanthin	601.4	12.4	0.998	1.2	2.1	91	99	0.11	7.6
cis-violaxanthin	601.4	17.5	0.993	1.3	2.0	102	102	0.12	19.6
all-trans-neoxanthin	601.4	13.2	0.997	3.6	3.4	78	104	0.30	13.3

[a] Lower limit of quantification: nanograms on-column.

calibration curves constructed on five different concentrations had good linearity ($r^2 = 0.981−0.999$). Each carotenoid extracted from citrus was analyzed within the linear range in the present study. The LLOQ of LC-MS was 10−394-fold lower than that of the UV−vis absorbance detection (**Table 3**). The recoveries of carotenoids, except for neoxanthin, were >84% (**Table 3**). The CV % for the recovery was low (<7%) in each carotenoid (data not shown). Thus, we added an internal standard, citranaxanthin, just before the LC-MS analysis to correct the response variation of the mass spectrometer because of the sufficient recoveries and slight CV observed in almost all carotenoids extracted. The intraday and interday precisions

of target carotenoids ranged from 1.0 to 4.8% and from 2.0 to 5.4%, respectively (**Table 3**). The accuracy ranged from 90 to 106% (**Table 3**). Matuszewski et al. suggested that the assessment of the matrix effect, which could severely impair the accuracy of quantification by ion enhancement or suppression to target compounds during the ionization process, is important when a homologous compound is utilized as an internal standard instead of a stable isotope-labeled compound (*28*). Therefore, the matrix effect was examined by comparing the slopes of the matrix-matched calibration curves. For example, in flavedo, the CVs of the slopes in abundant carotenoids, such as violaxanthin, β-cryptoxanthin, and phytoene, were 1.7, 1.3, and 2.7%,

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Table 4. Patterns of Seasonal Changes of Carotenoids[a] and Classification of Citrus Varieties on the Basis of the Patterns of Seasonal Changes of Phytoene, β-Cryptoxanthin, and Violaxanthin in Flavedo

| | patterns of seasonal changes clustered on the basis of carotenoid contents | | | | | | | | | | | | |
| | acyclic carotenoids | | | β,ϵ-carotenoids | | | β,β-carotenoids | | | | | | |
sample no.	PHY	PHF	ZCA	ACA	LUT	LUE	BCA	BCR	ZEA	ANT	VIO	NEO	cluster name[b]
A-1	L	L	L	L	L	L	M	L	M	L	L	L	FL-I
A-2	L	L	L	L	L	L	M	L	L	L	L	L	FL-I
B-1	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
B-2	M	M	H	H	M	H	M	M	L	M	H	L	FL-IV
B-3	M	M	H	L	L	H	M	M	L	L	L	L	FL-IV
C-1	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
C-2	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
C-3	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
C-4	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
C-5	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
C-6	L	L	L	L	L	L	M	L	L	L	L	L	FL-I
C-7	H	H	H	L	L	L	L	L	L	L	L	L	FL-II
D-1	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
D-2	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
D-3	L	L	L	L	L	L	L	L	M	L	L	L	FL-I
D-4	L	L	L	M	H	M	H	L	M	M	L	M	FL-I
D-5	L	M	M	L	M	M	L	L	L	M	L	L	FL-I
D-6	L	L	M	L	L	L	L	L	L	L	M	L	FL-III
D-7	L	L	L	L	L	L	L	L	L	M	M	L	FL-III
D-8	M	M	M	H	M	L	L	L	M	M	M	M	FL-IV
D-9	L	L	L	L	L	L	L	L	H	M	M	L	FL-III
D-10	L	L	L	L	M	L	H	L	M	L	L	L	FL-I
D-11	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
D-12	L	L	L	L	L	L	L	L	L	L	L	L	FL-I
D-13	L	L	L	M	L	L	M	L	M	L	L	L	FL-I
E-1	H	H	H	L	L	L	L	L	L	L	L	L	FL-II
E-2	L	L	L	L	M	L	L	L	L	L	L	L	FL-I
F-1	L	L	M	L	M	L	M	M	L	L	M	L	FL-IV
F-2	H	M	M	L	M	L	M	H	H	H	H	L	FL-V
F-3	L	L	L	M	H	M	M	L	L	L	L	H	FL-I
F-4	L	L	M	L	L	L	L	L	L	L	L	L	FL-I
F-5	L	L	M	L	H	M	M	M	H	H	H	L	FL-IV
F-6	L	L	M	H	H	M	H	H	H	H	H	H	FL-IV
F-7	M	M	M	HH	H	M	M	M	H	H	H	M	FL-IV
F-8	L	L	M	H	M	M	M	M	M	H	H	M	FL-IV
F-9	L	M	M	L	H	H	M	L	H	H	H	M	FL-III
F-10	H	H	HH	L	M	M	M	H	H	H	H	L	FL-V
F-11	M	M	M	M	M	L	M	M	L	M	M	L	FL-IV
F-12	L	L	L	L	L	L	M	L	H	H	H	L	FL-III

[a] Abbreviations of carotenoids: PHY, phytoene; PHF, phytofluene; ZCA, ζ-carotene; ACA, *all-trans*-α-carotene; LUT, *all-trans*-lutein; LUE, *all-trans*-lutein epoxide; BCA, *all-trans*-β-carotene; BCR, *all-trans*-β-cryptoxanthin; ZEA, *all-trans*-zeaxanthin; ANT, *all-trans*-antheraxanthin; VIO, violaxanthin; NEO, *all-trans*-neoxanthin. [b] The clusters were classified on the basis of the scores for the patterns of seasonal changes in PHY, BCR, and VIO.

respectively. In juice sacs, the values were 4.0, 0.8, and 4.3%, respectively. Thus, it seems that the matrix effect was negligible in the present method because of the low CV of the slopes in the matrix-matched calibration curves (*28*). The concentration analyzed by this LC-MS method was close to that analyzed by HPLC with the UV−vis absorbance method (e.g., β-cryptoxanthin: 12.2 μg/g, LC-MS, and 12.7 μg/g, HPLC).

Diversity in Patterns of Seasonal Changes of Carotenoids in Citrus Varieties. The contents of carotenoids in the flavedo and juice sacs of 39 varieties were determined monthly. In the present study, a trace amount (under the limit of quantification) of lycopene was observed in red blush grapefruit. Neurosporene and γ-carotene were undetected. Thus, the data of three carotenoids were not shown in the present study. In violaxanthin (two isomers) and ζ-carotene (three isomers), the total amounts of isomers were used for cluster analysis. On the basis of the carotenoid contents, the patterns of seasonal changes from 39 varieties were clustered in each carotenoid.

In flavedo, the patterns of seasonal changes from 39 varieties were classified into three clusters, H, M, and L, in each carotenoid with the exception of α- and ζ-carotene (**Table 4** and **Figure 2**). In varieties belonging to the H cluster, the concentration of seasonal changes of carotenoids was kept higher than that in other varieties. In those belonging to the M cluster, the concentration was in the middle. In those belonging to the L cluster, the concentration was kept low. In α- and ζ-carotene, these three clusters and another one, in which the concentration of seasonal changes of carotenoids remained higher than that in the H cluster, were observed. Thus, the fourth cluster is shown as HH in **Table 4** and **Figure 2**. The concentrations of all acyclic carotenoids (phytoene, phytofluene, and ζ-carotene) increased continuously regardless of the cluster types until the end of the experimental period (**Figure 2**). The concentrations of α-carotene and lutein, a member of the β,ϵ-carotenoids (i.e., carotenoids with a β-ring and an ϵ-ring), gradually decreased in all cluster types during the experimental period. In contrast, the concentrations of lutein epoxide, another β,ϵ-carotenoid, increased temporarily in one cluster (H cluster) but did not change dramatically in others (**Figure 2**). The concentrations of β-carotene and neoxanthin, members of the β,β-carotenoids (i.e., carotenoids with two β-rings), decreased in all clusters during the experimental period. In contrast, the concentrations of other β,β-carotenoids, β-cryptoxanthin, zeaxanthin, antheraxanthin, and violaxanthin, increased gradually and reached their maxima
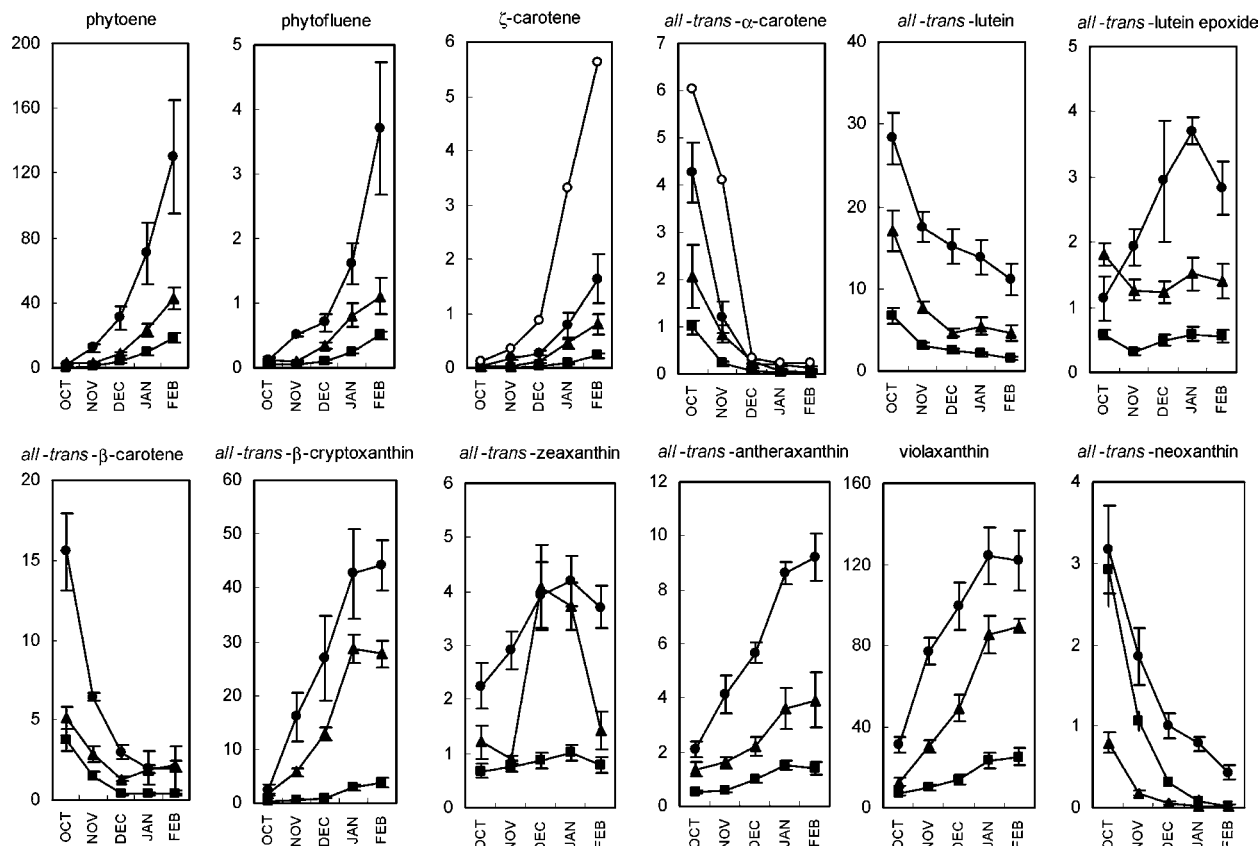
Figure 2. Seasonal changes of carotenoids for the HH cluster (○), H cluster (●), M cluster (▲), and L cluster (■) in flavedo. Values (micrograms per gram) are mean ± SE. Violaxanthin and ζ-carotene are the sum of isomers.

around January regardless of the cluster type (**Figure 2**). Subsequently, the concentrations of these carotenoids remained high or decreased.

In juice sacs, the patterns of seasonal changes were also classified into three clusters, H, M, and L, in all carotenoids, with the exception of α-carotene and neoxanthin (**Table 5** and **Figure 3**). In α-carotene and neoxanthin, the patterns of seasonal changes were classified into two clusters, H and L. In clusters H and M, a continuous increase was observed in almost all carotenoids (**Figure 3**). On the other hand, in the L cluster, no remarkable increase in carotenoids was observed.

Classification of Citrus Varieties on the Basis of Patterns of Seasonal Changes of Carotenoids. In flavedo, 39 varieties were classified into 5 clusters, FL-I, -II, -III, -IV, and -V (**Table 4**). The seasonal changes of carotenoids in each cluster are presented in **Figure 4**. In clusters FL-I, -III, -IV, and -V, the concentrations of lutein, a β,ϵ-carotenoid, were high in October and decreased with ripening. However, in cluster FL-II, no massive accumulation of lutein in October was observed. The concentrations of α-carotene and lutein epoxide, another β,ϵ-carotenoid, remained low in all clusters during the experimental period. The concentrations of phytoene, an acyclic carotenoid, increased markedly until the end of the experimental period in clusters FL-II and -V, although the increases were slight in other clusters. The concentrations of phytofluene and ζ-carotene, other acyclic carotenoids, remained low in all clusters. In clusters FL-II and -V, a massive accumulation of phytoene was observed even after the stage (around January) when β,β-carotenoids, such as violaxanthin and β-cryptoxanthin, reached their maximum concentrations. The concentrations of violaxanthin increased with ripening in all clusters. In clusters FL-III, -IV, and -V, the concentrations were higher than those in other clusters. In the concentrations of β-cryptoxanthin, distinct differences among

five clusters were observed. The concentrations in clusters FL-IV and -V were higher than those in other clusters. The concentrations of β,β-carotenoids, with the exception of violaxanthin and β-cryptoxanthin, remained low in all clusters. On the basis of these results, each cluster was characterized as follows: cluster FL-I was carotenoid-poor; cluster FL-II, phytoene-abundant; cluster FL-III, violaxanthin-abundant; cluster FL-IV, violaxanthin- and β-cryptoxanthin-abundant; and cluster FL-V, phytoene-, violaxanthin-, and β-cryptoxanthin-abundant.

In juice sacs, 39 varieties were classified into 4 clusters, JS-I, -II, -III, and -IV (**Table 5**). The seasonal changes of carotenoids in each cluster are presented in **Figure 5**. In clusters JS-II, -III, and -IV, the concentrations of lutein increased with fruit ripening, although the increase was not observed in JS-I. The concentrations of other β,ϵ-carotenoids remained low in all clusters. The concentration of phytoene increased markedly until the end of the experimental period in clusters JS-III and -IV. The concentrations of other acyclic carotenoids remained low in all clusters. The concentrations of violaxanthin increased with fruit ripening in JS-II, -III, and -IV. The concentrations in cluster JS-III were significantly higher than those in clusters JS-II and -IV. In β-cryptoxanthin, the concentration in cluster JS-IV was higher than that in other clusters. In cluster JS-IV, β-cryptoxanthin increased continuously even after the stage (around January) when violaxanthin reached its maximum concentration. The concentrations of β,β-carotenoids, with the exception of violaxanthin and β-cryptoxanthin, remained low in all clusters during the experimental period. As in the case in flavedo, each cluster in juice sacs was characterized as follows: cluster JS-I was carotenoid-poor; cluster JS-II, viola-xanthin-abundant at a middle level; cluster JS-III, violaxanthin-abundant at a high level and phytoene-abundant; and cluster

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Table 5. Patterns of Seasonal Changes of Carotenoids[a] and Classification of Citrus Varieties on the Basis of the Patterns of Seasonal Changes of Phytoene, β-Cryptoxanthin, and Violaxanthin in Juice Sacs

sample no.	acyclic carotenoids			β,ϵ-carotenoids			β,β-carotenoids						cluster name[b]
	PHY	PHF	ZCA	ACA	LUT	LUE	BCA	BCR	ZEA	ANT	VIO	NEO	
A-1	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
A-2	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
B-1	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
B-2	M	M	L	L	L	H	M	H	L	M	H	L	JS-IV
B-3	L	L	L	L	L	M	L	M	L	L	M	L	JS-II
C-1	L	L	L	L	L	L	M	L	L	L	L	L	JS-I
C-2	L	L	L	H	L	L	M	L	L	L	L	L	JS-I
C-3	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
C-4	L	L	L	L	L	L	L	L	M	L	L	L	JS-I
C-5	L	L	L	H	M	M	L	L	M	M	M	L	JS-II
C-6	L	L	L	L	L	M	L	L	L	L	M	L	JS-II
C-7	M	M	M	L	L	M	L	M	M	M	M	L	JS-IV
D-1	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
D-2	L	L	L	L	L	H	L	L	L	M	M	L	JS-II
D-3	L	L	L	L	L	M	L	L	M	M	M	L	JS-II
D-4	L	L	L	L	M	M	L	L	L	L	M	L	JS-II
D-5	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
D-6	L	L	L	H	H	H	L	L	M	M	H	L	JS-III
D-7	L	L	L	H	M	M	L	L	M	M	H	L	JS-III
D-8	M	M	L	H	H	H	M	M	H	H	H	H	JS-III
D-9	L	L	L	L	L	H	L	L	M	M	M	L	JS-II
D-10	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
D-11	L	L	L	M	M	L	L	L	L	M	L	L	JS-II
D-12	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
D-13	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
E-1	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
E-2	L	L	L	L	L	M	L	L	M	M	L	L	JS-I
F-1	M	M	M	L	L	M	M	H	L	M	M	L	JS-IV
F-2	M	M	M	H	L	M	M	H	H	M	M	L	JS-IV
F-3	L	L	L	L	M	L	L	L	L	L	M	H	JS-II
F-4	L	L	L	L	L	L	L	L	L	L	L	L	JS-I
F-5	H	H	H	L	L	H	H	H	M	M	M	L	JS-IV
F-6	H	H	M	H	M	M	H	H	M	M	H	L	JS-IV
F-7	M	H	M	H	M	H	H	H	H	H	M	L	JS-IV
F-8	M	H	M	H	L	H	H	H	H	M	M	L	JS-IV
F-9	M	M	M	H	H	M	L	L	M	M	H	L	JS-III
F-10	H	H	M	H	H	M	H	H	M	M	M	H	JS-IV
F-11	M	H	M	H	M	H	H	H	H	H	H	L	JS-IV
F-12	L	L	L	H	H	H	L	L	M	M	H	L	JS-III

[a] Abbreviations of carotenoids: PHY, phytoene; PHF, phytofluene; ZCA, ζ-carotene; ACA, *all-trans*-α-carotene; LUT, *all-trans*-lutein; LUE, *all-trans*-lutein epoxide; BCA, *all-trans*-β-carotene; BCR, *all-trans*-β-cryptoxanthin; ZEA, *all-trans*-zeaxanthin; ANT, *all-trans*-antheraxanthin; VIO, violaxanthin; NEO, *all-trans*-neoxanthin. [b] The clusters were classified on the basis of the scores for the patterns of seasonal changes in PHY, BCR, and VIO.

JS-IV, violaxanthin-abundant at a middle level and phytoene- and β-cryptoxanthin-abundant.

Relationship in Characteristics of Carotenoid Accumulation between Flavedo and Juice Sacs. The 39 varieties are organized in **Table 6** on the basis of the clusters in both flavedo (FL-I, -II, -III, -IV, and -V) and juice sacs (JS-I, -II, -III, and -IV). With the exception of Tengu (C-7), the varieties classified into clusters FL-I (carotenoid-poor) and -II (phytoene-abundant), in which the concentrations of β,β-carotenoids were lower than those in other clusters in flavedo, belonged to clusters JS-I (carotenoid-poor) and -II (violaxanthin-abundant at a middle level), in which the concentrations for β,β-carotenoids were lower than those in other clusters in juice sacs. These varieties did not massively accumulate β,β-carotenoids in either flavedo or juice sacs. With the exception of Iyo (D-9), varieties classified into cluster FL-III (violaxanthin-abundant) in flavedo belonged to cluster JS-III (violaxanthin-abundant at a high level and phytoene-abundant) in juice sacs. These varieties accumulated large amounts of violaxanthin without a distinct increase in β-cryptoxanthin in both flavedo and juice sacs. With the exception of Rangpur lime (B-3) and Tankan (D-8), varieties classified into clusters FL-IV (violaxanthin- and β-cryptoxan-

thin-abundant) and -V (phytoene-, violaxanthin-, and β-cryptoxanthin-abundant) in flavedo belonged to cluster JS-IV (violaxanthin-abundant at a middle level and phytoene- and β-cryptoxanthin-abundant) in juice sacs. These varieties accumulated large amounts of β-cryptoxanthin in both flavedo and juice sacs.

DISCUSSION

LC-MS Method for Carotenoid Quantification in Citrus Fruit. HPLC with photodiode array detection has been widely used in carotenoid analysis (*14−18*). However, several citrus carotenoids on the basic route of the carotenoid biosynthesis could not be quantified using a single HPLC analysis because of the complicated carotenoid composition in citrus fruit, insufficient sensitivity of the detector, and interference by impurities in the extract (*13*). In the present study, an LC-MS method was developed to quantify 18 carotenoids on the basic route of the carotenoid biosynthesis in plants (**Table 3**). The linear range of the calibration curves in each carotenoid was wide enough to analyze the citrus extracts of many varieties at different stages of fruit ripening. The lower limit of LC-MS
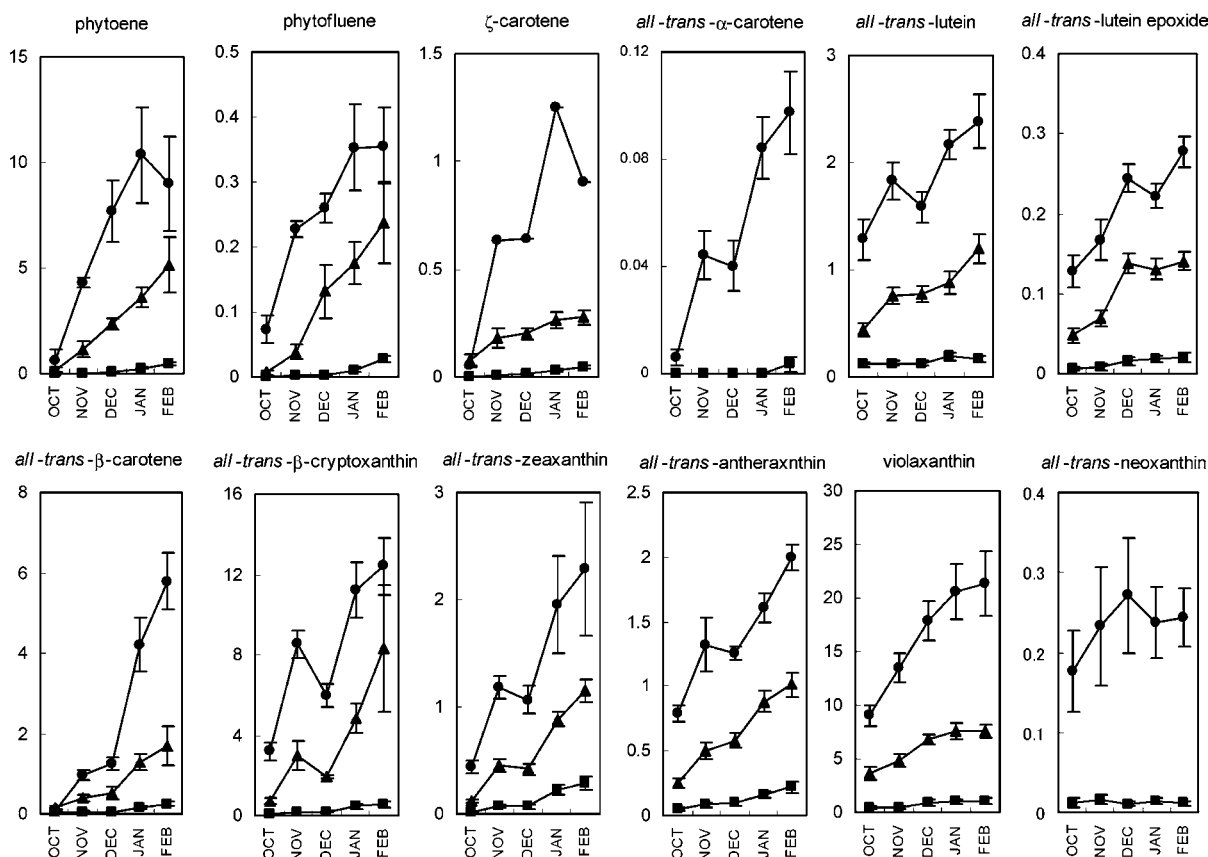
Figure 3. Seasonal changes of carotenoids for the H cluster (●), M cluster (▲), and L cluster (■) in juice sacs. Values (micrrograms per gram) are mean ± SE. Violaxanthin and ζ-carotene are the sum of isomers.

was 10−394-fold lower than that of the photodiode array detection. This method made it possible to analyze the citrus carotenoids on the basic routes of the carotenoid biosynthesis by a single injection.

In the present study, citranaxanthin was used as an internal standard. A previous study reported that citranaxanthin (C$_{33}$-apocarotenone) existed in citrus fruits (*31*). However, subsequently, citranaxanthin was found to be an artifact formed from β-apo-8′-carotenal during the saponification of citrus carotenoids in the presence of a small amount of acetone (*27*). Several citrus fruits are known to contain not only C$_{40}$-carotenoids, such as carotene (e.g., phytoene) and xanthophylls (e.g., violaxanthin), but also citrus-specific C$_{30}$-apocarotenoids, such as β-citraurin, β-citraurinene, and β-apo-8′-carotenal (*1*). Therefore, acetone was not used during the extraction and saponification procedure in the present study to avoid the formation of artifactural citranaxanthin.

Using this method, the seasonal changes of carotenoids on the basic routes of the carotenoid biosynthesis were analyzed in both flavedo and juice sacs of 39 citrus varieties at 5 different times during fruit ripening. The method developed in the present study was a sensitive, specific, and quick way to quantify carotenoids in plant.

Characteristics of Carotenoid Accumulation in Flavedo. Previous studies have shown that the pathway change from the synthesis of β,ε-carotenoids to that of β,β-carotenoids occurred with the transition of the peel color from green to orange on the basis of the level in gene expression for lycopene ε-cyclase and the content of lutein in the flavedo of Satsuma mandarin, Valencia orange, and Lisbon lemon (*13*) and in that of Navelate orange (*11*). In the present study, the concentrations of lutein, a β,ε-carotenoid, were high in the early mature stage (October)

and, subsequently, decreased with ripening in clusters FL-I, -III, -IV, and -V (**Figure 4**). Another β,ε-carotenoid, α-carotene, also decreased with ripening, although the concentration of α-carotene was much lower than that of lutein (**Figure 4**). In contrast, the concentrations of β-cryptoxanthin and violaxanthin, which are β,β-carotenoids, were low in the early mature stage and subsequently increased with fruit ripening (**Figure 4**). These results suggested that the pathway change occurred not only in the four citrus varieties shown in the previous papers (*11, 13*) but also in varieties belonging to clusters FL-I, -III, -IV, and -V. In two varieties, Teng (C-7) and Yuzu (E-1), belonging to cluster FL-II (a phytoene-abundant cluster), no distinct pathway changes were observed during the experimental period because the concentrations of the β,ε-carotenoids remained low throughout the experimental period (**Figure 4**). In addition, the concentrations of β,β-carotenoids in these varieties remained as low as those of cluster FL-I (a carotenoid-poor cluster). On the other hand, the concentrations of phytoene, phytofluene, and ζ-carotene were high in Teng and Yuzu (**Table 4**). It seemed that, in these varieties, suppressed desaturation by ζ-carotene desaturase resulted in the absence of pathway changes and abundant accumulation in acyclic carotenoids during the experimental period.

After the pathway changes, a massive increase in violaxanthin was observed in clusters FL-III, -IV, and -V. In clusters FL-IV and -V, β-cryptoxanthin also increased distinctly. These increases in β,β-carotenoids reached their maximum concentration around January. In cluster FL-V, a massive accumulation of phytoene was observed and continued after the concentrations of β,β-carotenoids reached their maximum. In cluster FL-II, a massive accumulation of phytoene was also observed after the increase in violaxanthin, although the concentration of viola-
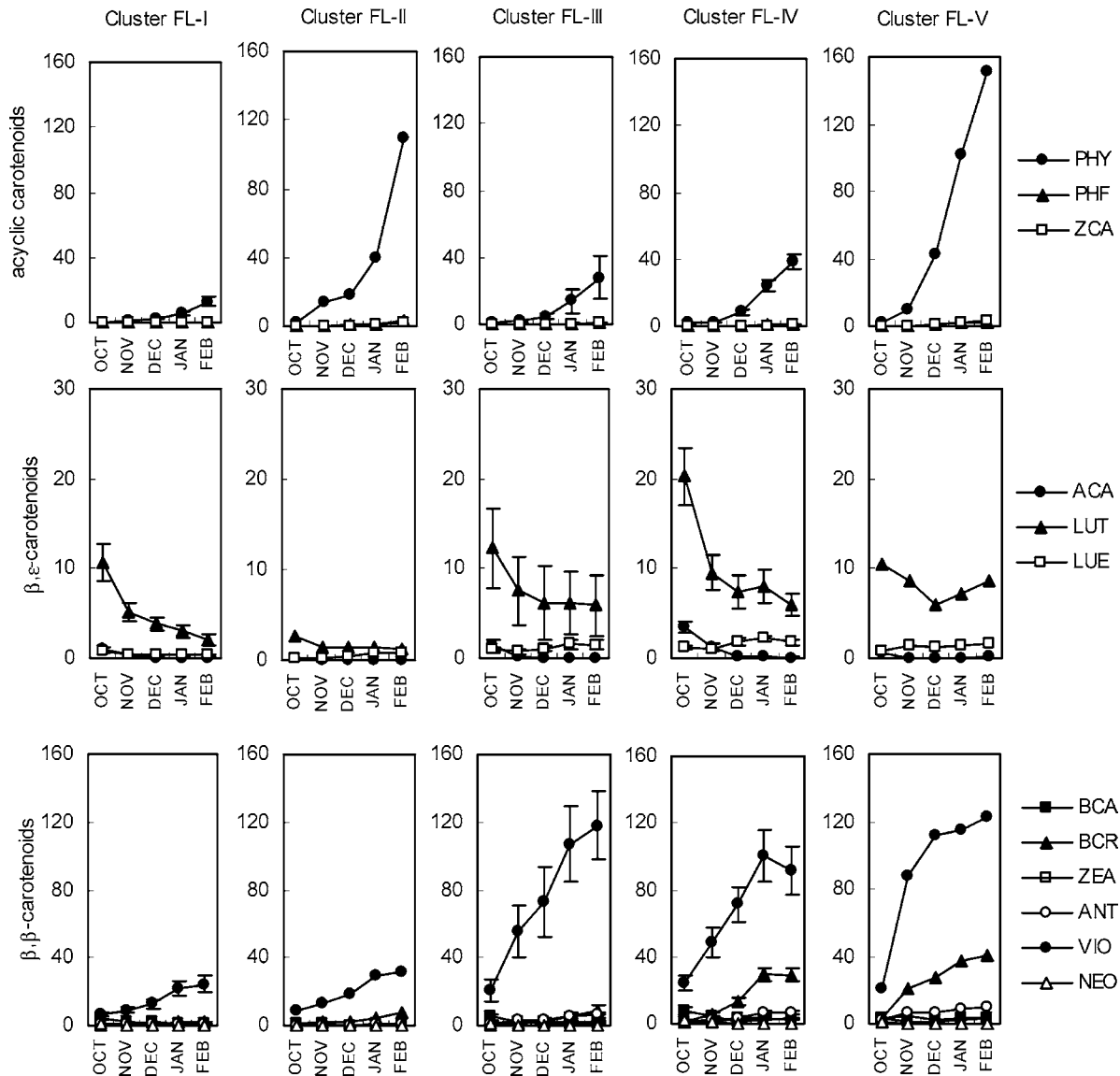
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Figure 4. Seasonal changes of carotenoids in each cluster classified on the basis of the patterns of seasonal changes of phytoene, β-cryptoxanthin, and violaxanthin in flavedo. PHY, phytoene; PHF, phytofluene; ZCA, ζ-carotene (sum of isomers); ACA, *all-trans*-α-carotene; LUT, *all-trans*-lutein; LUE, *all-trans*-lutein epoxide; BCA, *all-trans*-β-carotene; BCR, *all-trans*-β-cryptoxanthin; ZEA, *all-trans*-zeaxanthin; ANT, *all-trans*-antheraxanthin; VIO, violaxanthin (sum of isomers); NEO, *all-trans*-neoxanthin. Values (micrograms per gram) are mean $\pm$ SE.

xanthin was low in these varieties. In addition, in clusters FL-III and -IV, phytoene accumulated after the increase in violaxanthin, although the concentrations of phytoene were low. These results suggested that β,β-carotenoid accumulation preceded phytoene accumulation. Kato et al. (*13*) previously indicated that the accumulation of β,β-carotenoids preceded the accumulation of phytoene in the flavedo of Satsuma mandarin with a decrease in the transcript levels of phytoene desaturase. Rodrigo et al. (*11*) also observed that phytoene accumulated progressively until sufficient coloration was obtained in the flavedo of Navelate orange. The present study shows that phytoene accumulation after the increase in β,β-carotenoids was obvious not only in Satsuma mandarin and Navelate orange but also in many citrus varieties, with the exception of carotenoid-poor varieties (cluster FL-I). Thus, in flavedo, many citrus varieties, except for carotenoid-poor and phytoene-abundant varieties, massively accumulated β,ϵ-carotenoids, β,β-carotenoids, and phytoene, in that order. Moreover, in flavedo, no distinct difference in the accumulation order among β,β-carotenoids was observed.

Characteristics of Carotenoid Accumulation in Juice Sacs. Previously, Kato et al. (*13*) showed that no pathway change occurred during their experimental period in the juice sacs of Satsuma mandarin, Valencia orange, and Lisbon lemon. In the present study, lutein increased with ripening in clusters JS-II, -III, and -IV (**Figure 5**). Other β,ϵ-carotenoids, α-carotene and lutein epoxide, also increased with ripening, although the concentrations of α-carotene and lutein epoxide were much lower than that of lutein (**Figure 3**). These results suggest that no pathway change from the synthesis of β,ϵ-carotenoids to that of β,β-carotenoids was observed in the juice sacs of many citrus varieties.

In the present study, massive violaxanthin accumulation was observed in clusters JS-II, -III, and -IV. In addition, β-cryptoxanthin increased massively in cluster JS-IV. Phytoene increased in clusters JS-III and -IV. In cluster JS-IV, the concentration of violaxanthin was already high in October. In contrast, β-cryptoxanthin and phytoene were at the basal levels in October and, subsequently, increased continuously. In addition, in cluster JS-III, the concentration of violaxanthin was
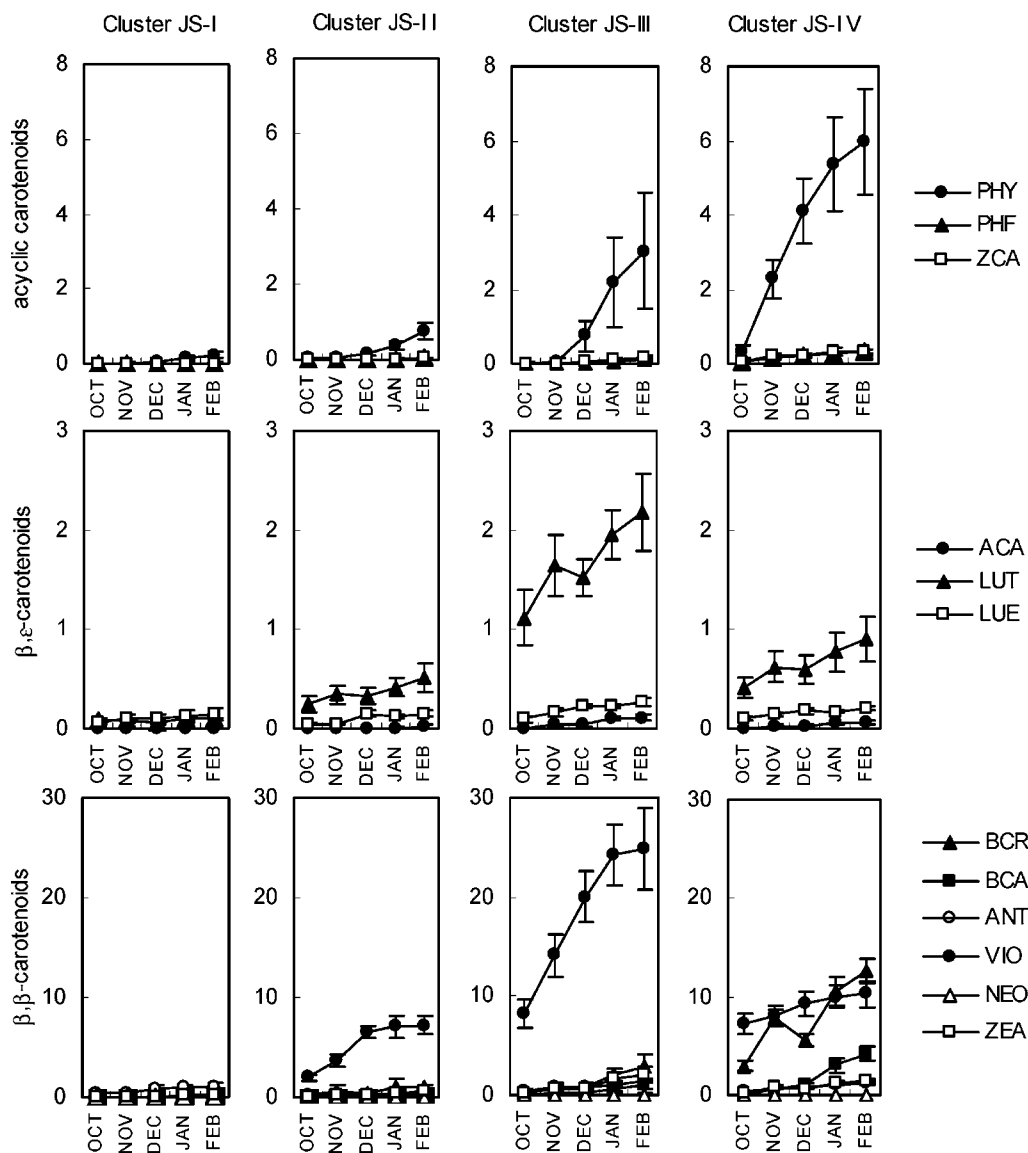
Figure 5. Seasonal changes of carotenoids in each cluster classified on the basis of the patterns of seasonal changes of phytoene, β-cryptoxanthin, and violaxanthin in juice sacs. PHY, phytoene; PHF, phytofluene; ZCA, ζ-carotene (sum of isomers); ACA, *all-trans*-α-carotene; LUT, *all-trans*-lutein; LUE, *all-trans*-lutein epoxide; BCA, *all-trans*-β-carotene; BCR, *all-trans*-β-cryptoxanthin; ZEA, *all-trans*-zeaxanthin; ANT, *all-trans*-antheraxanthin; VIO, violaxanthin (sum of isomers); NEO, *all-trans*-neoxanthin. Values (micrograms per gram) are mean ± SE.

already high in October. The increase in phytoene started in December. These results showed that, in juice sacs, the accumulation of violaxanthin was superior to that of other carotenoids at an early stage of maturation, with the exception of carotenoid-poor varieties (cluster JS-I). The accumulation of violaxanthin was followed by β-cryptoxanthin or phytoene accumulation at a later stage of maturation in many citrus varieties (clusters JS-III and -IV). The result that violaxanthin accumulation was followed by phytoene accumulation in juice sacs was consistent with the results obtained in the present and previous studies (*11, 13*) in flavedo. In contrast, the result that violaxanthin accumulation was followed by β-cryptoxanthin accumulation was unique in juice sacs. Lee et al. (*32*) also showed a distinct difference in the accumulation order among β,β-carotenoids in the juice of Hamlin orange. Thus, in juice sacs, the accumulation order among β,β-carotenoids was observed. Violaxanthin accumulation was followed by β-cryptoxanthin or phytoene accumulation in violaxanthin- and phytoene-abundant and violaxanthin-, phytoene-, and β-cryptoxanthin-abundant varieties.

In the present study, only a trace amount of lycopene was observed in red blush grapefruit during the experimental period. The flesh of the red blush grapefruit from our institute was light yellow, not pink, during the fruit-bearing period. Red-fleshed varieties of citrus, such as Star Ruby grapefruit, are known to accumulate lycopene in juice sacs in maturity (*12, 33*). The reason that lycopene did not accumulate in red blush grapefruit harvested in our country is uncertain. An environmental factor for growth, such as temperature, might have had an influence on the accumulation of lycopene in this cultivar.

Classification of Citrus Varieties and Relationship between Flavedo and Juice Sacs in Carotenoid Accumulation. Previously, Goodner et al. (*19*) demonstrated that mandarins, oranges, and their hybrids were quite distinct in their juice because of β-cryptoxanthin. Fanciullino et al. (*12*) investigated the carotenoid composition of citrus juice and showed that two carotenoids, *cis*-violaxanthin and β-cryptoxanthin, were strong determinants in citrus classification. In the present study, the carotenoid profiles in both flavedo and juice sacs were analyzed,

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Table 6. Classification of Citrus Varieties on the Clusters in both Flavedo (FL-I, -II, -III, -IV, and -V) and Juice Sacs (JS-I, -II, -III, and -IV)

cluster name (carotenoid profile)[a]	cluster FL-I (carotenoid-poor)	cluster FL-II (PHY-abundant)	cluster FL-III (VIO-abundant)	cluster FL-IV (VIO- and BCR-abundant)	cluster FL-V (PHY-, VIO-, and BCR-abundant)
cluster JS-I (carotenoid-poor)	A-1 A-2 B-1 C-1 C-2 C-3 C-4 D-1 D-5 D-10 D-12 D-13 E-2 F-4	E-1			
cluster JS-II (VIO-abundant at a middle level)	C-5 C-6 D-2 D-3 D-4 D-11 F-3		D-9	B-3	
cluster JS-III (VIO-abundant at a high level and PHY-abundant)			D-6 D-7 F-9 F-12	D-8	
cluster JS-IV (VIO-abundant at a middle level and PHY- and BCR-abundant)		C-7		B-2 F-1 F-5 F-6 F-7 F-8 F-11	F-2 F-10

[a] Abbreviations in the carotenoid profile: PHY, phytoene; BCR, β-cryptoxanthin; VIO, violaxanthin.

and citrus varieties were classified on the patterns of seasonal changes of violaxanthin, β-cryptoxanthin, and phytoene. This result shows that violaxanthin and β-cryptoxanthin were important determinants in citrus classification in both flavedo and juice sacs (**Table 6**). Moreover, this result suggested that, in each variety, the carotenoid profiles between flavedo and juice sacs on the basis of the concentration in violaxanthin and β-cryptoxanthin were similar to each other with the exception of a few varieties. For example, carotenoid-poor varieties in flavedo were also carotenoid-poor in juice sacs, and β-cryptoxanthin-abundant varieties in flavedo were also β-cryptoxanthin-abundant in juice sacs.

In the present study, most varieties in section Acrumen, including many mandarins, were classified into a β-cryptoxanthin-rich division. In contrast, oranges, such as Trovita and Washington navel (D-6 and -7, varieties in section Aurantium), were classified into the violaxanthin-rich division. This result, in which mandarins and oranges were divided according to the concentration of violaxanthin and β-cryptoxanthin, agreed with previous findings (*12*, *19*). Many varieties in sections Limonellus (including limes), Citrophorum (Eureka lemon), and Cephalocitrus (including grapefruits and pummelos) were separated from oranges and mandarins in our classification because of low β,β-carotenoids in lime, lemon, grapefruit, and pummelo. The result that varieties of low β,β-carotenoids were separated from oranges and mandarins agreed with that in a previous paper (*12*). In the present study, *C. limonia* (Rangpur lime and Limonia) was classified into the β-cryptoxanthin-rich division in flavedo, although this species belonged to the Citrophorum section. A previous paper (*12*) also showed that *C. limonia* was separated from other

species in the Citrophorum section, such as *C. limon*. Nicolosi et al. (*34*) suggested that *C. limonia* was a hybrid crossed with mandarin. Thus, it seems that *C. limonia* possessed β-cryptoxanthin-rich characteristics derived from mandarin. Fanciullino et al. (*12*) reported a similar idea. Thus, our results showed that, in each variety, the carotenoid profiles between flavedo and juice sacs were similar on the basis of the concentration of violaxanthin and β-cryptoxanthin, with the exception of a few varieties. Moreover, the results of the classification used in the present study almost agreed with those in a previous paper (*12*).

In conclusion, a highly sensitive LC-MS method was developed for the simultaneous quantification of 18 carotenoids on basic routes of the carotenoid biosynthesis in plants. With this method, the seasonal changes of citrus carotenoids in flavedo and juice sacs of 39 varieties during fruit ripening were investigated. In flavedo, the carotenoid profiles were diverse among citrus varieties, which were classified into five clusters. However, the order of carotenoid accumulation of β,ϵ-carotenoids, β,β-carotenoids, and phytoene was common among many citrus varieties, except for carotenoid-poor and phytoene-abundant ones. No distinct difference in the accumulation order among β,β-carotenoids was observed in flavedo. In juice sacs, the carotenoid profiles were diverse among citrus varieties, which were classified into four clusters. A distinct difference in the accumulation order among β,β-carotenoids was observed in juice sacs. Violaxanthin accumulation preceded β-cryptoxanthin accumulation in violaxanthin-, phytoene-, and β-cryptoxanthin-abundant varieties. In each variety, the carotenoid profiles between flavedo and

juice sacs based on the concentration in violaxanthin and β-cryptoxanthin were similar, with the exception of a few varieties.

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